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13. ABSTRACT (Maximum 200 Words)

The fps/fes proto-oncogene encodes a 92 kDa protein tyrosine kinase. To understand the physiological function of Fps we have generated a knockout mouse line that lacks Fps expression. Fps-knockout female mice produce litters that gain weight more slowly than wildtype mice, and develop breast tumors more quickly than wildtype mice. These data suggest that Fps participates in regulating mammary gland development and tumorigenesis. To address these hypotheses we are examining the biological and biochemical function(s) of Fps in the mammary gland. To date, we have shown that Fps is expressed specifically in epithelial cells. Examination of Fps during different mammary stages has shown that expression begins to increase during pregnancy and reaches maximal levels during lactation. An upregulation of Fps tyrosine kinase activity displays a similar profile. This would suggest that Fps might regulate epithelial cell differentiation. However, morphological and histological analyses could not detect any obvious differences in the pattern or quantity of alveolar structures between wildtype and knockout mice. Examination of ducts during lactation indicates that knockout cells may have a distorted shape with respect to their apical surface being more extended into the lumen of the duct. The exact nature of this potential phenotype has not yet been fully characterized but it suggests that Fps may regulate an aspect of lactation.

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Introduction:

The fps/fes proto-oncogene encodes a 92 kDa protein tyrosine kinase. To understand the biological function of the Fps kinase we have generated a knockout mouse (fps-null) model that lacks the Fps protein. During the initial analysis of this mouse line two important observations were made. First, litters reared by fps-null mice gained weight significantly more slowly than wildtype mice, and was determined to be dependent upon the genotype of the mother. Second, using a breast cancer mouse model, breast tumors developed earlier in the fps-null genetic background compared to the wildtype background. These observations suggest that Fps has an important role in the normal development of the mammary gland and possibly as a suppressor of mammary tumor development. The overall purpose of this research proposal is designed to elucidate the biological and biochemical function(s) of Fps in the mammary gland by 1) identifying the morphological abnormalities associated with the loss of Fps expression, 2) identifying the signaling pathways in which Fps participates and 3) determining important interacting protein molecules and substrates that Fps regulates. It is anticipated that this research will show that Fps regulates one or more stages of mammary gland development, possibly the lactation stage, and may function in pathways or cell processes that inhibit tumor initiation and/or progression.

Body:

Objective #1: Determine the pattern and level of expression of the fps gene in the mammary gland.

#1A. Generate constructs for anti-sense RNA expression of Fps and an epithelial marker.

Progress: Constructs for mouse and human Fps have been generated and anti-sense RNA has been expressed. A plasmid for cytokeratin-18 has been obtained but has not yet been cloned into the appropriate expression plasmid.

#1B. Perform RNA *in situ* hybridization analysis on whole mammary glands and histological sections.

Progress: To determine the expression pattern of Fps multiple attempts were made on both tissue pieces and sections using two different RNA *in situ* protocols without any success. The reasons for this are unclear and this part of the objective has been temporarily put on hold. However, we have addressed the expression pattern by confocal microscopy. Using a Fps-specific antibody we have shown that Fps is present in cells lining the ducts and alveoli of the mammary gland. By comparing this to the pattern of E-cadherin fluorescence we were able to conclude that Fps is predominantly expressed in mammary epithelial cells (**Figure 1**). We cannot exclude low level Fps expression in other cell types but it is comparatively very low or completely absent.

#1C. Northern blot analysis on RNA from different stages of pregnancy and lactation to obtain a quantitative expression profile of the *fps* gene.

Progress: Three mammary glands were harvested from each stage of development. Stages include: Virgin 3, 6, 9 weeks, Pregnancy day 6, 12, 18, Lactation day 4, 12, Involution day 2, 7. Total RNA has been isolated from all of the samples and frozen for future analysis. My lab is currently testing a new detection method for northern blots that avoids using radioactivity. Therefore, this objective has not been completed but should be in the next 2-4 months. In my lab a similar detection method has been developed for Southern blot analysis which is very sensitive and shortens the length of exposure time required to detect an adequate signal. This method involves generating a probe containing DIG-labeled nucleotides which can then be detected using an anti-DIG antibody and fluorescence detection system. If this method proves to be unuseful then radio-labeled probes will be generated and the experiment will be repeated. This method has been used successfully in the past for Southern and northern analyses but is not the preferred method of choice.

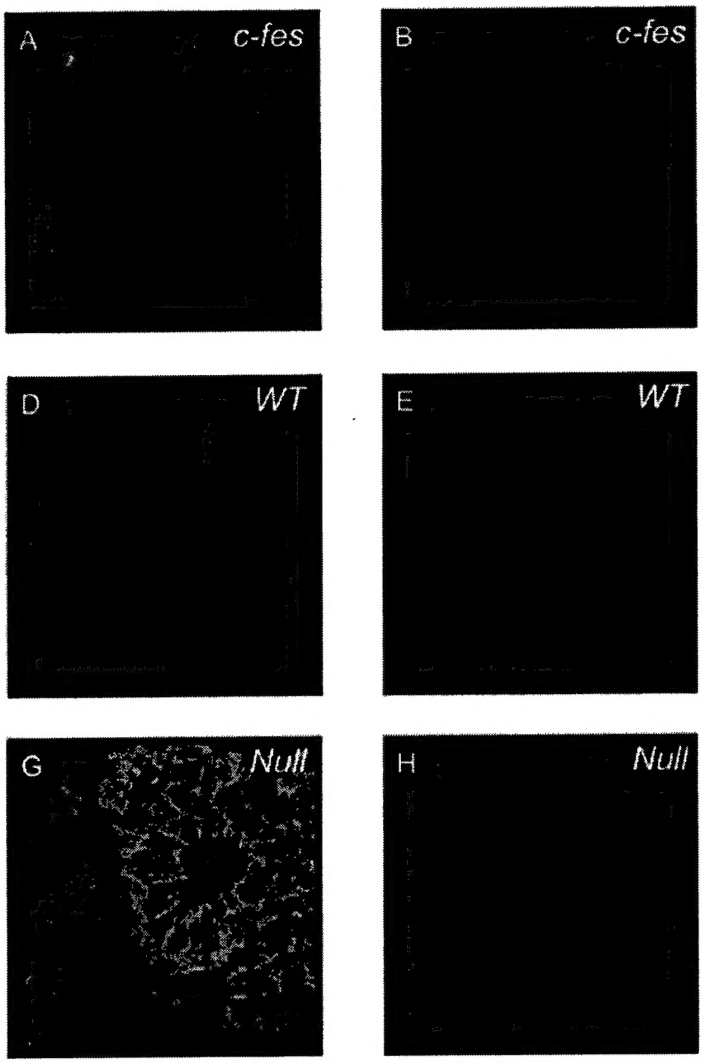

Figure 1. Subcellular localization of the fps tyrosine kinase in mammary gland epithelial cells. Fps protein was visualized using the FpsLQ antibody and an Alexa 633 secondary antibody. E-cadherin was detected with an E-cadherin specific antibody and an Alexa 488 secondary antibody. Mammary glands from c-fes, wildtype and fps-null mice were analyzed. The c-fes mice are a transgenic mouse line that contain 15-20 copies of a human fps transgene. This tissue was used to provide a greater signal to noise ratio for detecting Fps protein.

To supplement this data, parallel samples from the same mice were harvested for protein analysis. Tissue samples were homogenized in standard cell lysis buffer and then quantitated by Bradford assay. For each stage equal amounts of protein were taken from each of the three tissue samples and combined to compensate for any mouse to mouse variation in protein levels. Then, for each developmental stage, equal amounts of protein were analyzed by western blotting using an antibody which detects Fps and the related Fer protein with equal ability. This antibody (FpsQE) was used because the expression of Fer is ubiquitous and constant, and as such serves as a good internal control when assessing the amount of protein in each sample. This experiment has shown that Fps levels are very low during virgin stages and then increase as pregnancy progresses, culminating in its highest level of expression during lactation, followed by a decrease as involution occurs (**Figure 2**). Immunoprecipitation experiments have shown that the kinase activity of Fps mirrors its expression profile with high activity occurring during lactation (**Figure 3**). These data suggest that Fps plays an important role in some aspect of lactation that is compromised in the Fps knockout mice.

Objective #2: Perform mammary gland morphological analyses.

#2A. Whole-mount mammary gland staining analysis.

Progress: Mammary glands were harvested from virgin, mid-pregnancy and lactating mice and subjected to whole-mount staining to view the ductal structures. In brief, glands were fixed, incubated with acetone to remove fat, dehydrated in ethanol, stained with hematoxylin, washed, dehydrated in ethanol, cleared in xylene and mounted with permount solution. Originally, mice from wildtype, knockout and hyperactive-Fps mice were to be analyzed. However, the hyperactive-Fps mice were not used after it was determined that there was no difference between them and the wildtype, and the hyperactive-Fps possessed little tyrosine kinase activity based on phosphotyrosine analysis. Glands from both wildtype and knockout mice were extensively analyzed. Unfortunately, ductal structures from the knockout mice did not differ from the wildtype to any great extent at each of the three stages (**Figure 4**). Computer software to measure/count ductal branching and alveolar buds has recently been purchased. This program will be used over the next two to four months to provide a quantitative assessment of mammary development in both wildtype and knockout mammary glands.

#2B. Histological analysis of mammary gland sections.

Progress: Wildtype and knockout mammary glands from mid- and late-pregnant and lactating mice were processed and H&E stained. The tissue processing was performed by a technician in the Department of Pathology at Queen's University who specializes in this type of work. Basically, the tissue was fixed in formalin, embedded in paraffin, sectioned and then stained using a standard Hematoxylin/Eosin protocol. Glands from mid-pregnant mice do not possess a large number of ducts and were not analyzed thoroughly. Glands from late-pregnant mice did not show any obvious differences. Because Fps is most active during lactation this stage was the primary focus. There is no obvious difference in the overall ductal pattern or extent of ductal penetration within the gland (**Figure 5**). However, when individual ducts are compared there appears to be a difference in the fps-knockout mice. Specifically, the epithelial cells appear to have a distorted shape, with the apical surface protruding into the ductal space (**Figure 6**). Possibly these cells have a reduced rate of secretion of milk components that causes the observed distention. I am currently performing an experiment where the glands are allowed to fully expand with milk prior to harvest. This should standardize all variables and allow for an accurate analysis. Samples from the same tissue are going to be compared using an electron microscope to view the internal structures of the secretory cells. This should provide a conclusive assessment of any sub-cellular difference between the two genotypes.

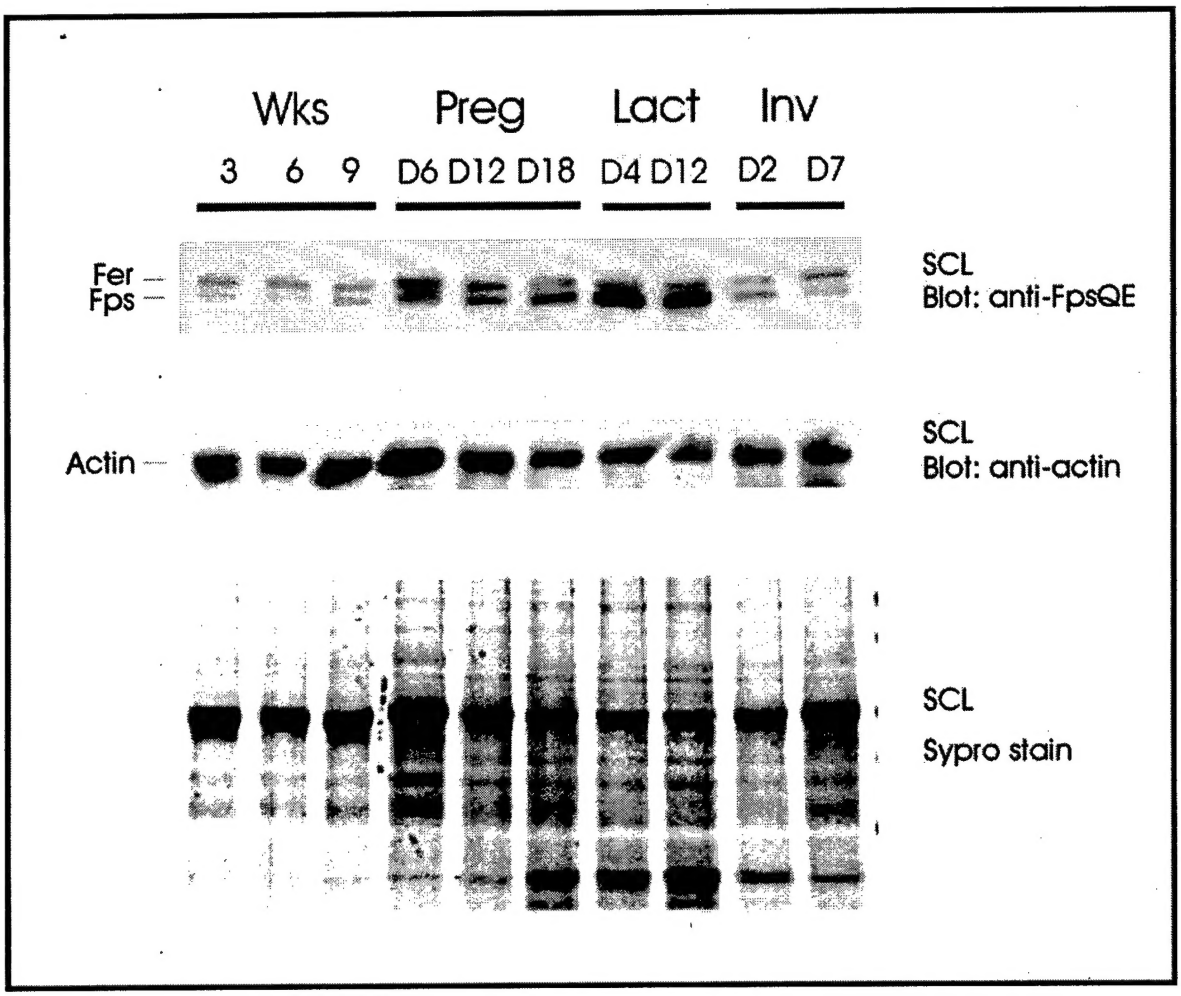

Figure 2. Expression profile of the Fps tyrosine kinase during mammary gland development. Mammary glands were harvested during different stages of development and subjected to western analysis. The antibody (FpsQE) was used to detect both Fps and Fer. The relative amount of protein loaded for each sample is demonstrated in the anti-actin and Sypro stain blots. Developmental stages are Virgin (week 3, 6, 9), Pregnancy (day 6, 12, 18), Lactation (day 4, 12), Involution (day 2, 7).

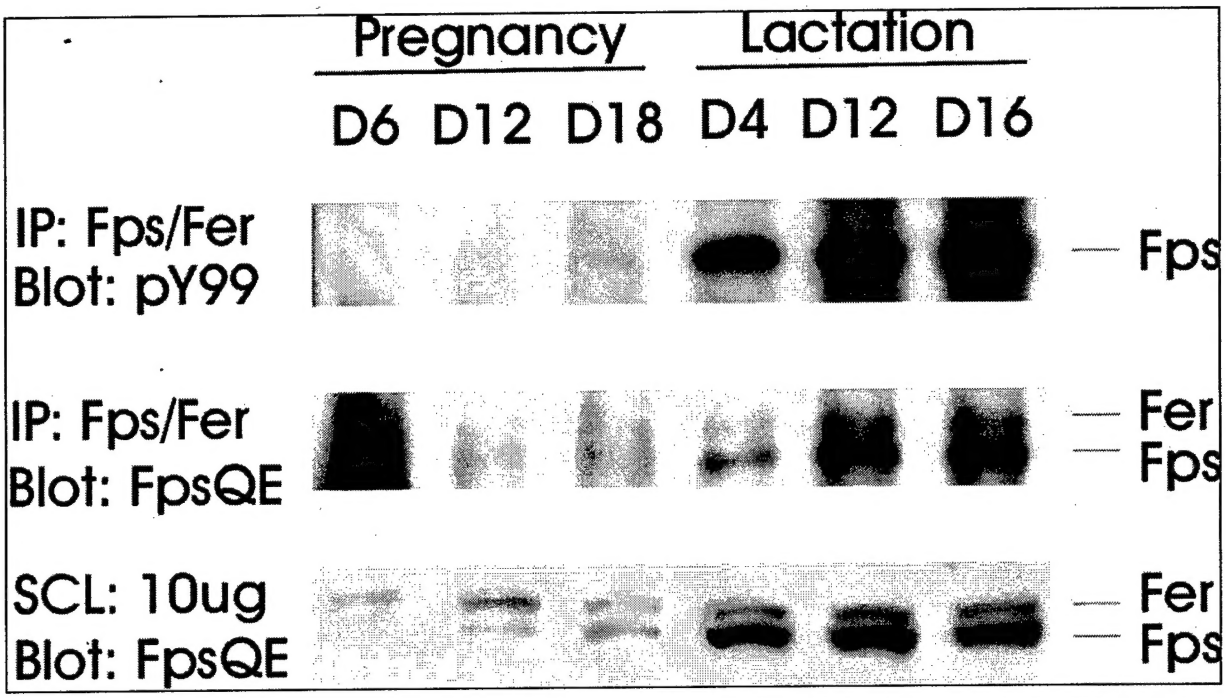

Figure 3. Activation of the Fps tyrosine kinase in the mammary gland during lactation. Fps and Fer proteins were immunoprecipitated from lysates of mammary glands at different stages of pregnancy and lactation with the anti-FpsQE antibody. Anti-phosphotyrosine (PY99) and anti-FpsQE antibodies were used to assess Fps phosphorylation and quantity, respectively.

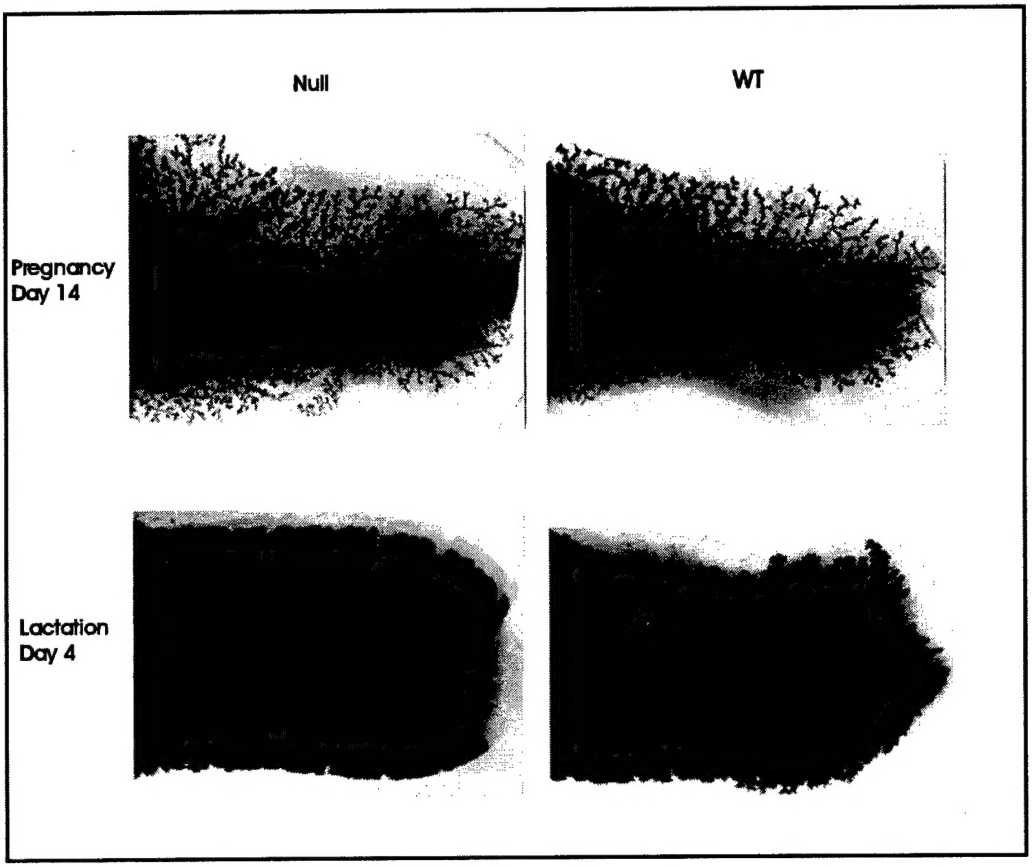

Figure 4. Wholemount hematoxylin staining of wildtype and fps-null mammary glands during pregnancy and lactation. Glands were harvested at the specified stage and stained with hematoxylin to identify the epithelial ductal structures.

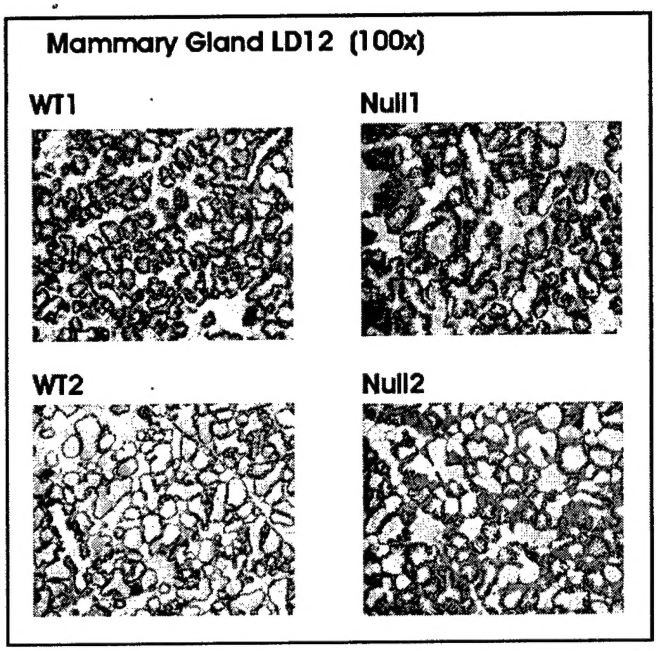

Figure 5. Histological analysis of lactating mammary glands from wildtype and fps-null mice. Tissues were sectioned, stained with hematoxylin and eosin and photographed at low magnification.

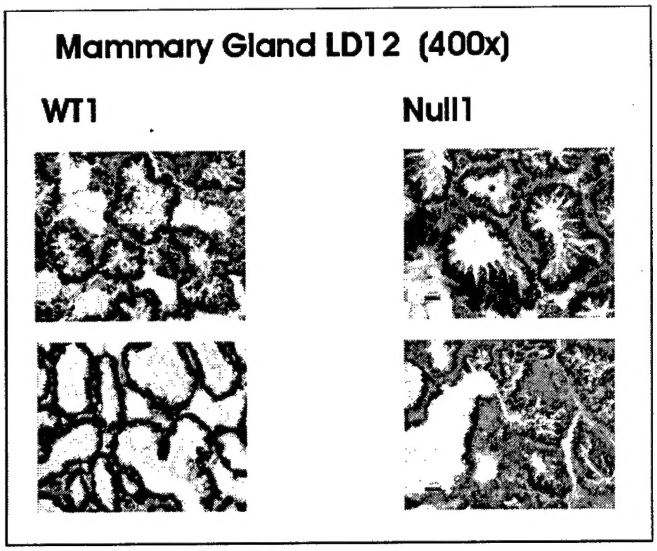

Figure 6. Histological analysis of lactating mammary glands from wildtype and fps-null mice. Tissues were processed as above and photographed at high magnification.

Objective #3: Generation of mammary epithelial cell lines from transgenic mice with different *fps* genetic backgrounds for *in vitro* studies.

#3A. Isolation of primary epithelial cells and growth in culture.

Progress: Epithelial cells have been isolated from mid-pregnancy mammary glands for both wildtype and fps-knockout mice. After much trial and error we have found a protocol which allows for good yield and expansion of the epithelial cell fraction prior to growth on tissue culture plates. This method involves growing the clumps of epithelial cells in a collagen gel for 2 to 3 weeks in the presence of EGF and insulin. Not only does this promote epithelial growth but it inhibits fibroblast growth. Photos of the 3-dimensional epithelial structures grown in collagen and the subsequent growth of epithelial cells on culture plates are provided (**Figure 7a, 7b**). Currently, the fps-knockout cells are growing in culture, and the wildtype cells are being expanded in a collagen gel before being plated. Within 1-2 months we should have good healthy cells growing in culture.

#3B and C. Immortalization of primary cells and characterization of cell lines to confirm their epithelial lineage.

Progress: None to date. We are planning on allowing the primary cells from #3A to immortalize naturally instead of introducing an immortalizing agent (as originally described). Primary epithelial cells can spontaneously immortalize after 3-5 passages in culture after initially being grown in collagen gel. It may take several more passages and purifications to establish a pure epithelial cell line. This may take up to 3-4 more months to finish this task. The lines will be characterized for expression of epithelial markers, like cytokeratin-18 and E-cadherin, using both confocal microscopy and FACS analysis.

Objectives #4 and #5 are designed to be accomplished over the next two years.

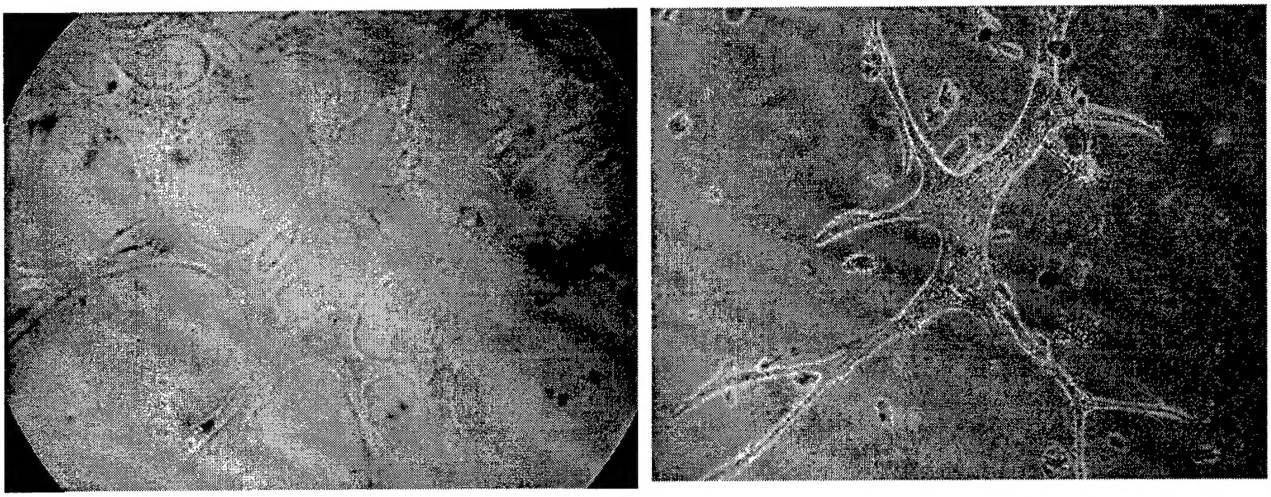

Figure 7a. Mammary gland epithelial cells from mid-pregnant mice grown in collagen gel. After 2-3 weeks masses of epithelial cells begin to grow and form 3-dimensional ductal structures. The left photo is a low maaginfication view, and the right photo is a more magnified image of an individual structure. Both wildtype and fps-null cells produce similar structures.

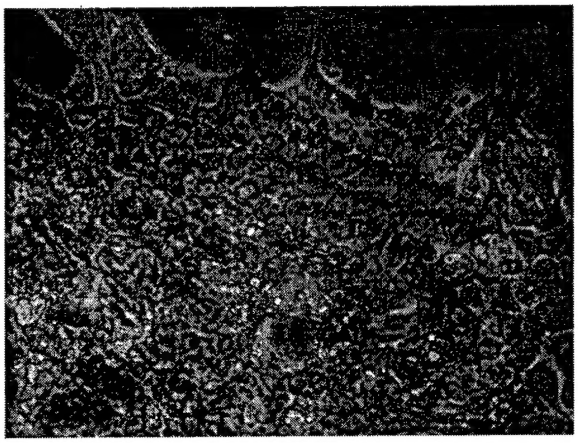

Figure 7b. Mammary gland epithelial cells growing on a culture plate. After growth in a collagen gel for 3 weeks the cell clumps were recovered by digestion of the collagen and then allowed to adhere to the plate and continue proliferating.

Key Research Accomplishments:

- Fps is expressed in mammary gland epithelial cells in mice.
- Fps expression is upregulated during pregnancy and reaches maximal levels during lactation.
- Fps tyrosine kinase activity is highly upregulated during lactation.
- Loss of Fps expression may result in morphological alterations of epithelial cells during lactation that result in altered milk production.

Reportable Outcomes:

1. Poster presentation at the 45th Annual Meeting of the American society of Hematology 2003. Title: Characterization of Fps Tyrosine Kinase Function in the Mouse Mammary Gland During Lactation. Authors: Peter Truesdell, Ralph Zirngibl and Peter Greer.

Conclusions:

Over the past year we have shown that the Fps tyrosine kinase is expressed in epithelial cells within the mammary gland. Analysis of its developmental expression profile indicates that there is low-level mammary expression of Fps in unmated mice that begins to increase as pregnancy progresses. There is a further increase at parturition, after which time expression is maintained at maximal levels until involution occurs when it decreases to pre-pregnant levels. Also, the tyrosine kinase activity displays a similar profile. This suggests that Fps regulates some aspect of mammary gland function, probably during lactation. Analysis of mammary ductal structures indicates no obvious structural defects in Fps-knockout mice. However, there may be a difference in the shape of the knockout epithelial cells during lactation where they have a more elongated extended apical surface. The significance of this is not presently known but it is possible that a loss of Fps results in a decreased rate of secretion of milk components that, in turn, leads to a build up of intracellular material and a "swelling" of the cell.

On its own this information is not overly impressive in terms of a role in breast cancer development. The important information from these experiments is that Fps is expressed in the mammary gland and it is active. Its developmental regulation suggests that Fps does indeed have a role in mammary gland function. And, the fact that Fps is expressed in the mammary gland lends supports to our hypothesis that Fps regulates breast tumor development. It will be important to determine what protein(s) Fps interacts with and phosphorylates to elucidate the precise role of Fps in the mammary gland. Our future experiments involve the development of cell lines from each of these mouse models so that specific signaling pathways can be activated and then assessed for alterations in the absence of Fps. We will also examine the components of the adherens junctions to see if the stoichiometry of the adherens junction components is altered in the knockout mice. Together, these experiments are expected to provide strong support of a role for Fps in breast development and tumorigenesis.

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